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THE REGULATION OF HAEMOCYANIN OXYGEN AFFINITY DURING EMERSION OF THE CRAYFISH *AUSTROPOTAMOBIUS PALLIPES*

III. THE DEPENDENCE OF Ca^{2+}–HAEMOCYANIN BINDING ON THE CONCENTRATION OF L-LACTATE

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SUMMARY

The binding of Ca^{2+} to the haemocyanin of the crayfish *Austropotamobius pallipes* was investigated. The amount of bound Ca^{2+} was determined using an ultrafiltration technique to produce haemocyanin-free solutions, the Ca^{2+} concentration of which could then be compared with that of the original, unfiltered solution. Any difference between the two values would indicate the amount of calcium bound by haemocyanin.

The effect of L-lactate on Ca^{2+} binding was investigated by determining the amount of bound ion at different concentrations of L-lactate. In addition, oxygen equilibrium curves were constructed for some of the solutions to verify that the haemocyanin oxygen affinity remained sensitive to L-lactate and to determine whether the haemocyanin was functionally similar to that used in previous investigations.

With 17 mmol l^{-1} total Ca^{2+} and approximately 1 mmol l^{-1} L-lactate the number of Ca^{2+} binding sites was estimated to be between eight and nine per haemocyanin molecule. Without taking into account the formation of calcium lactate, the observed dependency of Ca^{2+}–haemocyanin binding on L-lactate concentration could best be described by the equation: $Ca^{2+}/Hc = 8{\cdot}64 - 0{\cdot}32[lactate^-]$.

A 'worst case' estimate for maximum calcium lactate formation, assuming Ca^{2+} to be the only counterion available to lactate, altered the relationship slightly to: $Ca^{2+}/Hc = 8{\cdot}65 - 0{\cdot}35[lactate^-]$.

INTRODUCTION

During emersion the concentrations of calcium and L-lactate ions in the haemolymph of the crayfish *Austropotamobius pallipes* increase significantly (Taylor & Wheatly, 1981; Morris, Tyler-Jones, Bridges & Taylor, 1986*a*). It has, in addition,

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been shown that these changes in Ca^{2+} and L-lactate affect the oxygen affinity of the crayfish haemocyanin (Hc) in an agonistic manner (Morris, Tyler-Jones & Taylor, 1986*a*). Implicit in these reports was the suggestion that the binding, to the haemocyanin, of one of these ion species affected the binding of the other.

The increase in haemocyanin oxygen affinity caused by elevated $[Ca^{2+}]$ has been documented by a number of workers (Larimer & Riggs, 1964; Truchot, 1975; Weiland & Mangum, 1975; Arisaka & van Holde, 1979; Graham, Mangum, Terwilliger & Terwilliger, 1983), and the effect has been shown to be large in *A. pallipes* (Morris *et al.* 1986*a*,*b*). The potentiating effect of L-lactate on haemocyanin oxygen affinity (Truchot, 1980) has been found throughout the Crustacea although not in all species (Booth, McMahon & Pinder, 1982; Graham *et al.* 1983; Mangum, 1983*a*; Bridges, Morris & Grieshaber, 1984; Bridges & Morris, 1986; Morris & Bridges, 1986*a*). The direct binding of Ca^{2+} to haemocyanin has been demonstrated in several studies (e.g. Morimoto & Kegeles, 1971; Brouwer, Bonaventura & Bonaventura, 1978; Kuiper *et al.* 1979). There are, however, few investigations of the binding of L-lactate to haemocyanin (Graham *et al.* 1983; Johnson, Bonaventura & Bonaventura, 1984; Graham, 1985).

There is no evidence that Ca^{2+} and L-lactate in crustaceans interact directly or bind interactively to the haemocyanin (Graham *et al.* 1983). A study of turtle blood (Jackson & Heisler, 1982) indicates, however, that some calcium lactate should be formed in physiological solutions. In the present study, we determine to what extent the binding of Ca^{2+} to *A. pallipes* haemocyanin is dependent on the concentration of L-lactate in a species with a large calcium effect. It is suggested that the existence of such interdependent binding of these ions may result in a reduction of the observed potentiation of O_2 affinity by any one of these ions.

MATERIALS AND METHODS

Haemolymph procurement

The haemolymph used in these investigations was obtained by sampling (approx. 500 μl) from the pericardial space of several ($N > 10$) *Austropotamobius pallipes* (Lereboullet). The animals had been collected and maintained, and the blood handled as described previously (Morris *et al.* 1986*a*,*b*). As all the experimental material was to be dialysed, the ion concentrations in the pooled sample were not determined; instead, the dialysis solution was prepared on the basis of previous measurements made in this species (Morris *et al.* 1986*b*) and, except when explicitly mentioned, had the following composition (in $mmol\,l^{-1}$): NaCl, 181; KCl, 4·7; $CaCl_2$, 17; $MgCl_2$, 1·0; $NaHCO_3$, 3·0 (pH 7·9). In those cases where $CaCl_2$ was either 9 or 45 $mmol\,l^{-1}$, the concentration of NaCl was adjusted so that $[Cl^-]$ remained constant. These two values are representative of maximum and minimum $[Ca^{2+}]$ measured during a 24-h emersion period (Morris *et al.* 1986*a*). The use of 'hot' or 'cold' refers to the inclusion or absence of $^{45}Ca^{2+}$ in the experiment.

Preparation and measurement of cold solutions

Samples (2·5 ml) of pooled *A. pallipes* haemolymph were dialysed at 4°C for 24 h against a Ringer's solution (5 l, pH 7·9) containing 9, 17 or 45 mmol l^{-1} $CaCl_2$. The concentration of haemocyanin in the resulting solutions was determined spectrophotometrically by measuring the absorbance peak near 335 nm and calculating the concentration using an extinction coefficient of $2{\cdot}69E^{1\%}_{1cm}$. In subsequent experimental series protein concentration was also measured according to the method of Bradford (1976) (BioRad, protein test kit). This consistently produced values between 4 and 7 % greater than the calculated values for haemocyanin, indicating the presence of a non-haemocyanin protein in the haemolymph. The total concentrations of Ca^{2+} in the pooled sample and in all subsequent sub-samples in this series were determined in a u.v.-visible spectrophotometer (Kontron, Uvikon 710) using a colourimetric test (test kit 1028, Roche, Basel, Switzerland). After these determinations had been made, each dialysed haemocyanin solution was divided into two and centrifuged at 136 000 ***g*** for 30 min in a precooled rotor on an Air-Fuge (Beckman, California, USA) to pellet the haemocyanin. From each preparation (1 ml) 100 μl of supernatant Ringer's solution was removed and replaced by 100 μl of the same Ringer (pH 7·9) containing either 10 or 105 mmol l^{-1} L-lactate to give final concentrations in the remixed haemocyanin solutions of 1 or 10·5 mmol l^{-1}, respectively.

Each of the preparations was then introduced into the upper chamber of a Centricon 30 microconcentrator (Amicon GmbH, Witten, FRG). The microconcentrator is a styrene–acrylonitrile tube holding a polycarbonate membrane filter (M_r cut-off 30 000; haemocyanin $M_r > 68\,000$) at the mid-point. The tube can be capped at both ends and placed in a centrifuge. Centrifugation at 5000 ***g*** for 20 min forced approximately 25 % of the haemocyanin-free Ringer through the membrane into the lower chamber (filtrate). The amount was determined by weighing the collecting vessel before and after collection. A sub-sample was then taken from the filtrate to determine the total Ca^{2+} concentration ($[Ca^{2+}]_f$) for each preparation. The filtrate and the retained solution containing the haemocyanin were then proportionally recombined to produce a reconstituted sample and the $[Ca^{2+}]$ was measured. This determined how much, if any, Ca^{2+} had bound irreversibly to the microconcentrator parts. Taking these controls into account, it was then possible to compare the $[Ca^{2+}]_f$ of the haemocyanin solutions containing 10 and 1 mmol l^{-1} L-lactate. In addition, by subtracting $[Ca^{2+}]_f$ from the Ca^{2+} concentration measured in the original dialysed samples ($[Ca^{2+}]_d$), it was possible to estimate the bound Ca^{2+} in relation to [haemocyanin]. The procedure was repeated in triplicate.

Preparation and measurement of hot solutions

In the second series of determinations, four haemolymph samples were predialysed (24 h) against one of four Ringer's solutions (pH 7·9) containing approximately 0, 1, 5 and 10 mmol l^{-1} L-lactate and 17 mmol l^{-1} Ca^{2+}. A sample was removed from each dialysis for the determination of [haemocyanin] and [lactate$^-$],

and for the construction of oxygen equilibrium curves (see below). L-lactate was determined by the method of Gutmann & Wahlefeld (1974) modified according to Engel & Jones (1978).

$^{45}CaCl_2$ (Amersham Buchler GmbH & Co KG) was then added to the dialysis Ringer's solution so that 100 μl of Ringer contained 114000 d.p.m. (Ca^{2+} = 17 mmol l^{-1}). This involved the addition of 20 μl of $^{45}Ca^{2+}$ solution to each dialysis solution, which produced an insignificant change in $[Ca^{2+}]$, and dialysis was then continued for a further 12 h.

The resulting haemocyanin–$^{45}Ca^{2+}$ solutions were introduced into microconcentrators and treated as in the method described above. The $^{45}Ca^{2+}$ activities of the dialysis Ringer, dialysed blood, filtrate, retentate and reconstituted mixture were determined in a programmable scintillation counter (Beckman, Model LS 1801, Irvine, CA, USA) which corrected for quenching and counting efficiency. The quenching correction factor was installed by counting known $^{45}Ca^{2+}$ activities in the absence and presence of various amounts of *A. pallipes* haemocyanin. The difference between $[Ca^{2+}]_f$ in the filtrate and $[Ca^{2+}]_d$ in the dialysed haemolymph represented the calcium bound to haemocyanin.

Control experiment in the presence of EDTA

The introduction of EDTA (250 mmol l^{-1}) into the dialysed haemolymph prior to ultrafiltration resulted in the chelation of practically all Ca^{2+} (17 mmol l^{-1}) and thus negated any effect of the haemocyanin on the distribution of Ca^{2+}. All samples were generated and measured as described above.

Control experiments on the presence of a lactate effect

The oxygen equilibrium curves constructed for the haemocyanin dialysed against four different L-lactate concentrations in the second experimental series were prepared, using a spectrophotometric method, on samples in a diffusion chamber (Sick & Gersonde, 1969). This method, modified according to Bridges, Bicudo & Lykkeboe (1979) was that used previously in the investigation of Ca^{2+} and lactate effects on *A. pallipes* haemocyanin (Morris *et al.* 1986*a*,*b*). The half-saturation tension (P_{50}) and the cooperativity of O_2 binding at this tension (n_{50}) were calculated from regression equations for values between 25 and 75 % saturation (see Bridges *et al.* 1984).

Unless otherwise stated, all means are expressed ± S.D. The means of the sample groups were compared using Student's *t*-test, and significance levels are stated at appropriate points in the text and figure legends.

RESULTS

The distribution of Ca^{2+} between plasma and haemocyanin

In these experiments conducted without the use of $^{45}Ca^{2+}$, the concentration of haemocyanin was determined to be 50 ± 6 mg ml^{-1}. For the purposes of this

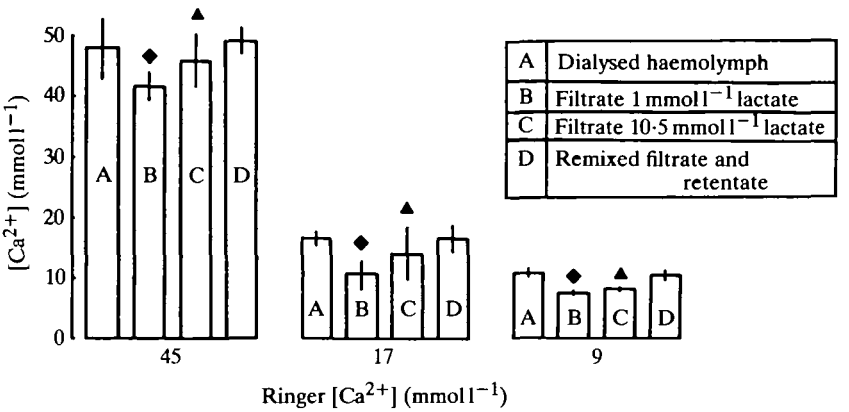

Fig. 1. The concentration of 'cold' calcium, measured spectrophotometrically, in haemolymph dialysed against Ringer's solution (pH 7·9) containing Ca^{2+} at 45, 17 and 9 mmol l^{-1} and no lactate, and in ultrafiltered solutions from dialysed *Austropotamobius pallipes* haemolymph with 1 and 10·5 mmol l^{-1} L-lactate. Also shown is the measured Ca^{2+} concentration in the solution obtained by proportionally recombining material retained in the microfilter unit with the filtrate. The diamonds indicate a significant difference between A and B, and the triangles between C and B ($P < 0{\cdot}01$). Error bars indicate ± 1 S.D. ($N = 3$).

experiment this was assumed to represent constant [haemocyanin]. The concentrations of Ca^{2+} measured in the various fractions from the microconcentrators are given in Fig. 1. Dialysis against the two extreme Ca^{2+} concentrations employed (9 and 45 mmol l^{-1}) resulted in haemocyanin solutions (A in Fig. 1) containing apparently greater concentrations of Ca^{2+} than had been present in the dialysis Ringer. A similar trend is observed at all three Ca^{2+} levels; the $[Ca^{2+}]$ of the filtered solution containing 1 mmol l^{-1} L-lactate is significantly lower than that of the dialysed haemocyanin solution. The magnitude of this difference is related to the absolute concentration of Ca^{2+}. It was not possible to demonstrate that the filtrate (C in Fig. 1) from preparations containing 10·5 mmol l^{-1} L-lactate contained concentrations of Ca^{2+} that differed significantly from those of the corresponding dialysed haemolymph (A in Fig. 1), but in all cases the $[Ca^{2+}]$ of the high-lactate solutions were higher than those in the low-lactate solutions (Fig. 1).

The proportional recombination of filtrate and retained material produced three solutions (D in Fig. 1), each of which contained Ca^{2+} at a similar concentration to that of the respective dialysed haemolymph (A in Fig. 1), thus indicating that there was no significant binding of Ca^{2+} to the microconcentrator. The amounts of Ca^{2+} calculated (see Materials and Methods) to be bound to the haemocyanin in the presence of 1 mmol l^{-1} L-lactate were 9·1, 8·70 and 4·8 mmol Ca^{2+} to one molecule of haemocyanin at $[Ca^{2+}]$ of 45, 17 and 9 mmol l^{-1}, respectively. Increasing the concentration of L-lactate to 10·5 mmol l^{-1} reduced the amount of bound Ca^{2+} by 63·5, 53·5 and 25 % for the same three Ca^{2+} concentrations, respectively.

Determination of binding using $^{45}Ca^{2+}$

The haemocyanin concentrations of the four preparations had a mean value of 53 ± 5 mg ml^{-1}. The different L-lactate concentrations of the four solutions are given in Figs 2 and 3. It is immediately apparent from Fig. 2 that the concentration of Ca^{2+} in the dialysed haemolymph preparations is significantly greater than that in the dialysis Ringer's solution (17 mmol l^{-1}). The filtrate, as hypothesized, contained Ca^{2+} at concentrations similar to that in the Ringer. Statistical analysis determined, however, that three of the four filtrates contained Ca^{2+} in amounts significantly greater ($P < 0{\cdot}05$) that that of the Ringer's solution. The retained haemocyanin solution held, in all four cases, Ca^{2+} at a higher concentration than the original dialysed haemolymph, which is consistent with the removal of 25% of the Ringer (filtrate) with a lower $[Ca^{2+}]$.

To clarify the role of L-lactate in influencing the binding of Ca^{2+} to haemocyanin, the contribution of bound Ca^{2+} to the $[Ca^{2+}]_d$ measured in dialysed haemolymph was corrected to a constant haemocyanin concentration of 50 mg ml^{-1} (Fig. 2). This was achieved by determining what the total calcium content of the solution was and adjusting this for a slightly higher or lower protein content. The amount of Ca^{2+} bound was also calculated as the mole ratio (Table 1; Fig. 2), which at near 0 mmol l^{-1} lactate approached 9 mmol Ca^{2+} mmol^{-1} haemocyanin. It can be seen

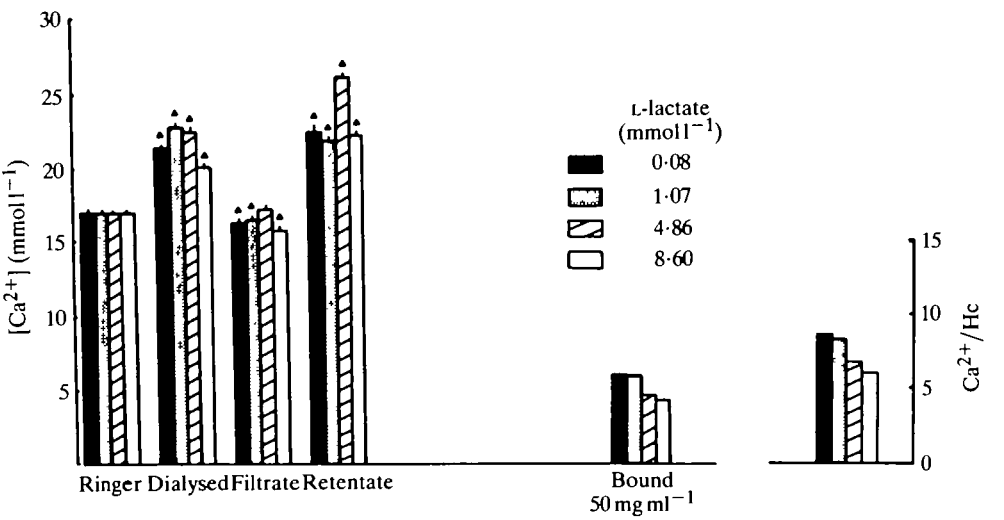

Fig. 2. Determinations of Ca^{2+} concentrations employing $^{45}Ca^{2+}$ measurement in four solutions containing L-lactate at different concentrations at pH 7·9. The first set of bars represents the concentration in the dialysis media and the second the Ca^{2+} concentration in the respective dialysed haemolymph solutions. The next two sets of bars illustrate the $[Ca^{2+}]$ in the four filtrates and retentates, respectively. The contribution of bound Ca^{2+} to the total $[Ca^{2+}]$ measured in the dialysed haemolymph normalized to 50 mg ml^{-1} haemocyanin concentration is also shown. On the right side of the figure is the mole ratio for Ca^{2+} : haemocyanin binding for the four different concentrations of L-lactate. The S.D. of the estimates are given and ▲ indicates a significant difference from the Ringer solution value ($P < 0{\cdot}05$) ($N = 6$).

Table 1. *The dependence on* L-*lactate*$^-$ *of the mole ratio of* Ca^{2+} *bound to the haemocyanin of* Austropotamobius pallipes *at pH 7·9 and 17 mmol* l^{-1} Ca^{2+}

[L-lactate$^-$] (mmol l^{-1})	mmol Ca^{2+}/mmol haemocyanin (1)	(2)	[calcium lactate] (mmol l^{-1})
0·08	8·61	8·61	0·02
1·07	8·55	8·53	0·33
4·86	6·63	6·47	1·55
8·60	6·16	5·88	2·50

(1) Determined assuming no calcium lactate formation; (2) adjusted on the assumption that calcium lactate reduces Ca^{2+} activity.

To the right of the table are the corresponding estimates of calcium lactate formation (see text for details).

from Table 1 that increasing L-lactate from 0·08 to 8·6 mmol l^{-1} brought about a reduction in bound Ca^{2+} of nearly 30%. A regression line was calculated for these data and can be described by the following relationship:

$$Ca^{2+}/Hc = 8{\cdot}64 - 0{\cdot}32[\text{lactate}^-] \quad (r = -0{\cdot}964),$$

where Ca^{2+}/Hc is the mole ratio of haemocyanin-bound calcium and [lactate$^-$] is measured in mmol l^{-1}.

In recognition of the fact that Ca^{2+} and L-lactate in solution will associate to an extent determined by the mass action of each species, the dependence of Ca^{2+}/Hc binding on [lactate$^-$] was further defined. A value of $k' = 19{\cdot}2$ l mol^{-1} (Jackson & Heisler, 1982) was used in the equation $[\text{calcium lactate}]/([Ca^{2+}] \cdot [\text{lactate}^-]) = k'$. Using the data shown in Fig. 1 it was possible to calculate a value for the dependency of Ca^{2+}/Hc binding on the activity of Ca^{2+} in respect of varying total $[Ca^{2+}]$. Using this information it was then possible to determine how Ca^{2+}/Hc binding would vary in respect of Ca^{2+} activity at a constant $[Ca^{2+}]$ of 17 mmol l^{-1} and varying levels of lactate. It was found that increasing [lactate$^-$] reduced Ca^{2+} activity and Ca^{2+}/Hc binding. Assuming that only calcium associated with lactate, the number of moles of Ca^{2+} bound per haemocyanin was reduced by 0·27 (when [lactate$^-$] was at most 8·6 mmol l^{-1}), due to the formation of approximately 2·5 mmol l^{-1} of calcium lactate. The following equation can be used to describe the refined relationship:

$$Ca^{2+}/Hc = 8{\cdot}65 - 0{\cdot}35[\text{lactate}^-] \quad (r = -0{\cdot}970).$$

Oxygen equilibria and the effect of L-*lactate*

The individual oxygen equilibrium curves are not presented, but the P_{50} and n_{50} values for each of the curves made using the solutions with four different [lactate$^-$] are shown (Fig. 3). The slope of the plots of $\log P_{50}$ *vs* pH and hence the Bohr effect (ϕ) did not differ significantly ($P < 0{\cdot}05$; analysis of covariance). The mean value for all the data was calculated as $\phi = 0{\cdot}46 \pm 0{\cdot}02$. The relationship between oxygen affinity and [lactate$^-$] could be expressed by the regression equation at pH 7·8: $\log P_{50} = 0{\cdot}589 - 0{\cdot}041 \log[\text{lactate}^-]$. This describes a very slight effect of [lactate$^-$]

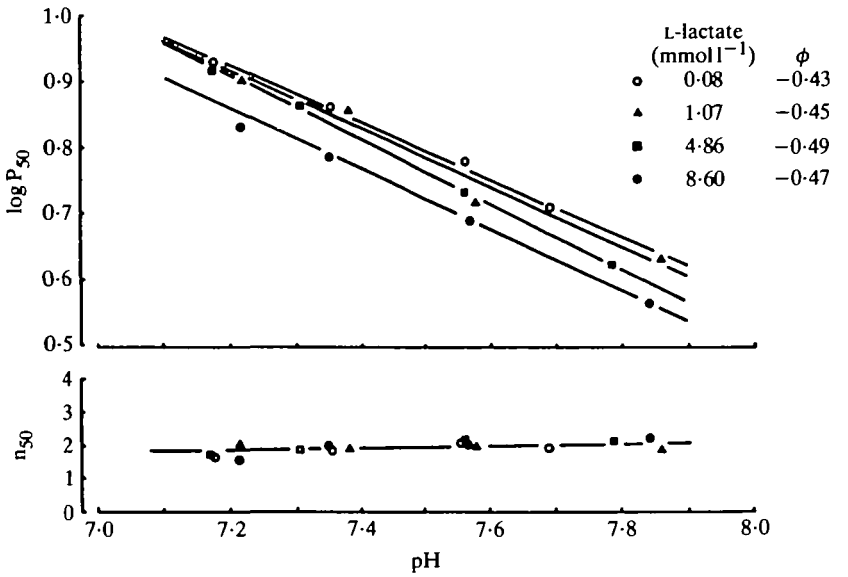

Fig. 3. The upper panel shows the pH dependence of oxygen affinity in dialysed solutions of *Austropotamobius pallipes* haemocyanin containing four different concentrations of L-lactate. The lower panel indicates the n_{50} values determined for the same haemocyanin solutions.

on oxygen affinity which was, however, still significant over the range $[\text{lactate}^-] = 0{\cdot}08-8{\cdot}6\,\text{mmol}\,l^{-1}$ (analysis of covariance, $P < 0{\cdot}05$). It was not possible to demonstrate a dependence of n_{50} on either the concentration of L-lactate or the pH within the physiological range.

Investigations of Ca^{2+} distribution in the presence of EDTA

The objective of this experiment was to determine whether calcium ions would distribute non-preferentially between the various microconcentrator fractions when haemocyanin-bound calcium was released. Although there was some variation in the amount of $^{45}Ca^{2+}$ in each of the dialysed solutions containing the four different concentrations of L-lactate, the same trend was observed throughout (Fig. 4). The differences in $[Ca^{2+}]$ previously seen in the comparison of dialysed haemolymph with the corresponding haemocyanin-free filtrate, and also with the retained material, were not observed in the presence of EDTA. The Ca^{2+} was indeed distributed evenly between all fractions, as would be expected for a small ion with no tendency to bind to the macromolecular components of the solution.

DISCUSSION

The physiological consequences of variation in the haemolymph concentration of Ca^{2+} and L-lactate for the oxygen affinity of *A. pallipes* haemocyanin have been discussed previously (Morris *et al.* 1986*a*,*b*). These investigations, although showing the interactive effect between calcium and lactate ions in potentiating haemocyanin

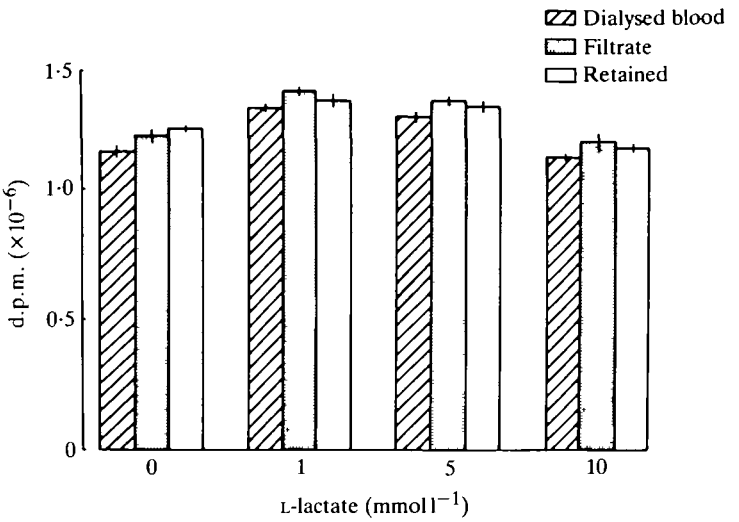

Fig. 4. The effect of the introduction of 250 mmol l^{-1} EDTA into four solutions of dialysed *Austropotamobius pallipes* haemolymph with L-lactate at four different concentrations and calcium at 17 mmol l^{-1}. The relative concentration of Ca^{2+} is expressed as the d.p.m. of $^{45}Ca^{2+}$ in the original dialysed preparations as well as in the filtrated and retained material resulting from each.

oxygen affinity, demonstrated neither the actual binding of Ca^{2+} to assembled haemocyanin polymers nor that the extent of this binding was dependent on the concentration of L-lactate.

The binding of Ca^{2+} to haemocyanin

Previous studies on the role of Ca^{2+} in crustacean haemocyanin function have considered primarily the assembly requirements for this ion (e.g. Morimoto & Kegeles, 1971; Kuiper *et al.* 1979) or have concentrated on the role of Ca^{2+} as an allosteric effector of the binding of O_2 to haemocyanin (e.g. Truchot, 1975; Brouwer *et al.* 1978; Miller & van Holde, 1981; Wheatly & McMahon, 1982; Mason, Mangum & Godette, 1983; Taylor, Morris & Bridges, 1985).

Kuiper *et al.* (1979) concluded that the number of calcium ions binding to *Panulirus interruptus* haemocyanin was pH-dependent and also that two types of oxygen-linked calcium-binding sites existed. These sites effected preferentially the T or the R state. Klarman & Daniel (1980) concluded that a major role of both Mg^{2+} and Ca^{2+} was to increase cooperative binding of O_2 to arthropod haemocyanin. Differences in the binding of Mg^{2+} and Ca^{2+} were, however, demonstrated for the haemocyanin of *Callianassa californiensis* by Arisaka & van Holde (1979), who identified approximately eight binding sites for Ca^{2+}, some of which had a very high affinity. The number of binding sites identified by Kuiper *et al.* (1979) in *Panulirus interruptus* was at least 10 at physiological pH.

Previous measurements of calcium binding by haemocyanins have usually been performed with isolated, purified haemocyanins using Tris buffers and EDTA

treatment initially to remove all Ca^{2+}. The present study used only native haemocyanin, with the only manipulation being a dialysis against a Ringer's solution of a similar composition to that of the plasma.

The estimated amount of Ca^{2+} bound by *A. pallipes* haemocyanin in the present study was dependent on the free Ca^{2+} concentration, although apparently less so at higher concentrations, as indicated by the cold experiments. This suggests that the haemocyanin of *A. pallipes* may, much like that of *C. californiensis* (Arisaka & van Holde, 1979), have some high-affinity and a number of lower-affinity Ca^{2+} binding sites. The Ca^{2+} sites that are lactate-labile may be regarded as low-affinity sites, whereas those still occupied by Ca^{2+} at high lactate concentrations may prove to be of higher affinity. Taking account of the results from both cold and hot experiments, a value of nine Ca^{2+}-binding sites per molecule of *A. pallipes* haemocyanin can be calculated, which is within the same order of magnitude as reported previously. In addition, using the same methods as in the present study, we showed that the haemocyanin of *Callinectes sapidus* had 4–5 Ca^{2+}-binding sites per molecule of haemocyanin (C. R. Bridges, in preparation); suggesting that nine sites for *A. pallipes* haemocyanin is not an overestimate.

L-lactate and its influence on Ca^{2+}–haemocyanin binding

The direct binding of L-lactate to crustacean haemocyanin has been more difficult to demonstrate. The steric nature of this molecule and the postulated binding site have been discussed (Graham *et al.* 1983; Mangum, 1983*b*; Graham, 1985; Bridges & Morris, 1986), but only Johnson *et al.* (1984) have determined the number of binding sites. In their study of *C. sapidus* haemocyanin, only 2·8 lactate-binding sites could be assigned per six oxygen-binding sites; this indicates a ratio of $<1{\cdot}0$ between L-lactate and the haemocyanin oxygen-binding site.

The present study determined that $\log P_{50}/\log [\text{lactate}^-]$ (the effect of L-lactate on the oxygen affinity of the *A. pallipes* haemocyanin) had a low value ($-0{\cdot}04$) in the presence of 17 mmol l^{-1} Ca^{2+}, confirming that the haemocyanin was functionally similar to the haemocyanin used in previous investigations. The value was, however, lower than that previously reported (Morris *et al.* 1986*b*), but it still represents a significant effect of L-lactate. Evidence has been presented that the magnitude of the lactate effect is dependent on $[Ca^{2+}]$ as is the allosteric effect of Ca^{2+} on $[\text{lactate}^-]$ (Morris *et al.* 1986*b*). It is now possible to conclude that this interdependence is due to interaction, not necessarily direct competition, in the binding of the two ions to the haemocyanin of *A. pallipes*. Graham *et al.* (1983) also attempted to demonstrate a similar phenomenon in solutions of *Cancer magister* haemocyanin, by measuring the change in the 'free' calcium concentration with a selective electrode in diluted samples after the addition of L-lactate. No change was detected in these experiments, which is interesting in view of the reduced activity of calcium at high lactate concentrations. *A. pallipes* haemocyanin also exhibits a high calcium sensitivity of C[illegible] binding (Morris *et al.* 1986*b*). Results for *Callinectes sapidus* haemocyanin

(C. R. Bridges, in preparation) suggest that lactate–calcium interactions may be reconciled with haemocyanins with a large calcium sensitivity of O_2 binding (Mason *et al.* 1983).

The formation of calcium lactate has been shown to occur in aqueous solutions to an extent defined by the association constant (Gosh & Nair, 1970; Jackson & Heisler, 1982). The value of $[Ca^{2+}/Hc]/[lactate^-]$ in respect of possible calcium lactate formation was estimated to be approximately −0·35. This is slightly different from the value of −0·32, calculated on the assumption of no formation of calcium lactate. The net result of this difference would be a reduction (at $[lactate^-] = 8{\cdot}6\,mmol\,l^{-1}$) in Ca^{2+}/Hc from 6·2 to 5·9. This refinement of the data represents a 'worst case' estimate, as it was not possible to determine the role of other ions such as Mg^{2+} and haemocyanin acting as counterions for L-lactate. Additionally, any formation of calcium lactate would not influence the distribution of the calcium in such a way as to simulate haemocyanin binding, since the Ca^{2+}–$lactate^-$ complex could easily pass the filter membrane and distribute non-preferentially, as did the EDTA–Ca^{2+} chelate.

This report demonstrates that increasing the concentration of L-lactate reduces Ca^{2+} binding. In one experiment, an increase in $[lactate^-]$ from 1 to $10{\cdot}5\,mmol\,l^{-1}$ reduced Ca^{2+} binding by approximately 50%. The second series, using $^{45}Ca^{2+}$ and increasing $[lactate^-]$ from 0·08 to $8{\cdot}60\,mmol\,l^{-1}$, resulted in a reduction of nearly 30%. The dependence of Ca^{2+} binding on the concentration of L-lactate was determined to have a coefficient of −0·35. This would suggest that an increase in $[lactate^-]$ of approximately $3\,mmol\,l^{-1}$ would be required to reduce by one the number of calcium ions bound to a haemocyanin molecule, when $[Ca^{2+}] = 17\,mmol\,l^{-1}$.

We cannot at present make firm conclusions about the mechanism of this interaction. Guesnon, Poyart, Bursaux & Bohn (1979) have discussed the binding of $lactate^-$ and Cl^- to human haemoglobin and whether this occurs at a common site. In this case the ions have at least the same charge sign, whereas it is more difficult to envisage competition at the same site between oppositely charged $lactate^-$ and Ca^{2+}. These authors (Guesnon *et al.* 1978) also demonstrated interaction between $lactate^-$, Cl^- and 2,3 diphosphoglycerate, which all have the same charge sign. A similar interaction, but with both positively and negatively charged ions, is observed in solutions of *A. pallipes* haemocyanin between lactate, calcium and urate ions (Morris, Bridges & Grieshaber, 1986; Morris & Bridges, 1986*b*). Manwell (1961) has suggested that the intramolecular interactions within the haemocyanin could be due to configurational changes rather than to the presence of any specific group within the protein. This argument may be applied to the interaction of Ca^{2+} and L-lactate. L-lactate induces a configurational change that decreases the affinity of the haemocyanin for Ca^{2+}. Kuiper, Zolla, Finazzi-Agro & Brunori (1981) have speculated that Ca^{2+}-binding sites on *P. interruptus* haemocyanin may be clustered, but there is no absolute requirement for lactate- and Ca^{2+}-binding sites to be in close proximity if the competitive effects are mediated by steric changes in protein

configuration. Such a conclusion would not be counter to the hypothesis of Johnson *et al.* (1984) that L-lactate binding sites may be between haemocyanin subunits.

The interdependence of the potentiating effects of lactate and Ca^{2+} binding to *A. pallipes* haemocyanin has been shown to have particular physiological consequences (Morris *et al.* 1986*a*). The binding of these ions potentiates O_2 affinity to varying extents, the effect increasing in a non-linear manner when the effector concentration is increased (see Tables in Bridges *et al.* 1984; Bridges & Morris, 1986). The reduction in the binding of one effector ion species brought about by the increased binding of the second effector species results in an apparent reduction in the potentiation due to the second effector. This creates the situation of apparent modulation of modulator function. This feature may be adaptive, however, as pointed out by Mangum (1983*b*) for *C. sapidus*. The simple addition of the separate effects of lactate and Ca^{2+} would increase haemocyanin oxygen affinity to an extent that O_2 extraction by the tissues would become impaired. In *A. pallipes*, preventing haemocyanin oxygen affinity from increasing to too great an extent, whilst maintaining the ability to modulate O_2 binding, may be a key adaptive feature.

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